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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Clinical data show that about 50% of the ER-positive patients respond to tamoxifen, the most widely used anti-estrogen. The factors that correlate with resistance/response to tamoxifen, or any other anti-estrogens, are largely not understood, but are likely to depend on the relative amounts of all ER forms present in the tumor tissue. Therefore, lack of knowledge of the correlation between the relative amounts of various ER isoforms with response is an important problem, because, without it, identification of patients who are most likely to respond to a particular anti-estrogen therapy is not possible. In the current study, we are testing the hypothesis that total ER isoform composition correlates with both ligand (estrogen or tamoxifen) and DNA binding properties using different cancer cell lines. Our results indicate that the three cell lines analyzed so far are distinct in their ER isoform composition. Although MCF-7 and T47D show similar binding to estrogen, they have distinct tamoxifen binding. ZR-75 cells have relatively high levels of ER alpha exon deletion variants and show lower binding to estrogen and relatively even less binding to tamoxifen. The DNA binding properties of the three cells lines are similar.

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INTRODUCTION

Recent studies have shown that breast tissues express both estrogen receptor (ER) alpha and beta, as well as over twenty isoforms of alpha and fifteen isoforms of beta (1-4). Breast cancer tissues have altered expression of both alpha and beta isoforms and are more heterogeneous in the composition of the various isoforms than are normal tissues (1-3,5). *In vitro* studies with individual receptors have demonstrated that the ERα, ERβ and the spliced variants have distinct ligand binding and transcriptional properties (6-9). It strongly follows that the relative proportion of ERα, ERβ and the spliced variants will result in different ligand- and DNA binding properties of the cell/tissue as a whole. Anti-estrogen therapy is the major treatment for patients who express estrogen receptor (ERα) in breast tumors. Clinical data show that only about 50% of the ER-positive patients respond to tamoxifen, the most widely used anti-estrogen. The factors that correlate with resistance/response to tamoxifen, or any other anti-estrogens, are largely not understood. Although, it is thought to be multifactoral, in all likelihood the response to a particular anti-estrogen therapy will depend on the relative amounts of all ER forms present in the tumor tissue. Therefore, lack of knowledge of the correlation between the relative amounts of various ER isoforms with response is an important problem, because, without it, identification of patients who are most likely to respond to a particular anti-estrogen therapy is not possible. In the current study, we are testing the hypothesis that total ER isoform composition correlates with both ligand (estrogen or tamoxifen) and DNA binding properties using different cancer cell lines. We have so far analysed three cells lines, MCF-7, T47D and ZR-75. Our results indicate all the three cell lines are distinct in their ER isoform composition. Although MCF-7 and T47D show similar binding to estrogen, they have distinct tamoxifen binding. ZR-75 cells have relatively high levels of ER alpha exon deletion variants and show lower binding to estrogen and relatively even less binding to tamoxifen. The DNA binding properties of the three cells lines are similar.

BODY

We have studied three estrogen positive, estrogen responsive cell lines: MCF-7, T47D and ZR-75. Cells were grown in estrogen-free, charcoal-stripped medium for 72 hrs, harvested with phenol-free trypsin and stored at -80° C. Cells were extracted with 20 mM HEPES, pH 7.6, 12% glycerol, 1 mM EDTA, 2 mM DTT, 400 mM KCl and protease inhibitors by three freeze - thaw cycles with frequent vortexing and then centrifugation at 27,000 X g for 20 min at 4°C.

Quantification of mRNAs of ERα (wild type), ERβ1 and ERβ2 and alternativley spliced variants of ERα

ER isoform mRNA copy numbers were determined by Real-Time PCR and are expressed as copy numbers per 10^{10} copies of GAPDH mRNA (Table 1). Each cell line had a distinct profile of ER composition, with T47D expressing the highest amount of total ER mRNAs and ZR-75 had the least. ERα (wild type) was the most prevalent in all three cell lines. ERβ1, ERβ2 and the deletion variants of ERα were present as a relatively small fractions when compared to ERα (wild type) in all cell lines (Table 2). However, ERα 2Δ was relatively abundant in the ZR-75 cells.

Ligand Binding Assays

For ligand binding assays, 50μg cell extracts were incubated with 0-2.5nM [^{3}H]-estrogen in the presence and absence of 200-fold excess of radioinert estrogen for 4hrs at 4°C. The bound ligands were separated by adding an equal volume of 50% hydroxyl apatite and specific binding was determined by subtracting nonspecific binding from total binding. The binding was analyzed according to Schatchard. **Competition Binding assays with estrogen and tamoxifen** were assessed with 50μg of cell extract by incubating with varying dilutions of cold estrogen or tamoxifen and saturating amounts of [^{3}H]-estrogen (1nM) for 4hrs at 4°C. After the incubation, bound ligands were separated and estimated by scintillation counting. The data was evaluated by nonlinear four parameter logistic model (using software from GraphPad Prism). The relative binding affinity of each competitor was calculated as the ratio of concentrations of estrogen vs competitor required to reduce the specific radioligand binding by 50% (=ratio of IC_{50} values).

All three cell lines showed similar binding affinity for estrogen (Figure 1), but ZR-75 extracts bound less total estrogen than extracts from the other two lines. This was likely due to a lower number of estrogen receptors in these cells. MCF-7 cell extracts bound a comparable amount of estrogen when compared to the T47D cells even though they had slightly fewer estrogen receptors.

In the competition binding assays, tamoxifen had a higher IC_{50} value (lower affinity) than the estrogen in all cases (Figure 2). When comparing cell lines, the MCF-7 cell extracts had the highest relative binding affinity for tamoxifen (when compared to estrogen binding affinity) and the ZR-75 extract the lowest (Table 3)

DNA Binding Assays

In vitro DNA binding was assessed by the gel mobility shift assay with labeled ERE oligonucleotides. For this twenty-two micrograms of protein from whole cell extracts were incubated for 20 min at RT in buffer containing 20 mM HEPES, pH 7.6, 12% glycerol, 1 mM EDTA, 2 mM DTT , 150 mM KCl, and 1 μg/20 μl Poly(dI•dC). Approximately 80 fmol of ERE oligonucleotide (Santa Cruz) labeled with approximately 50 nCi ^{32}P-containing ATP using a Ready-To-Go T4 Polynucleotide Kinase kit was added and the reaction further incubated for 30 min at RT. After the incubation period, the whole sample was loaded onto a prerun 5% non-denaturing acrylamide gel and electrophoresis was carried out at 200 V for 1 hr and 45 min. The gel was then dried and exposed to x-ray film. Competition assays were carried out as above, except 3 pmol of either cold ER consensus oligonucleotide (ERE) or GR consensus oligonucleotide (GRE) were included in the reaction mixture.

Among the three cell lines, T47D extracts showed somewhat more DNA binding than the extracts from the other two cell lines (Figure 3), but there was not much difference between them.

TABLE 1

ER ISOFORM mRNA COMPOSITION IN MCF-7, T47D AND ZR-75 CELL LINES (COPIES PER 10^{10} COPIES OF GAPDH)

Cell Type	ERα WT	ERα Exon 2Δ	ERα Exon 2-3Δ	ERα Exon 3Δ	ERα Exon 4Δ	ERα Exon 5Δ	ERα Exon 6Δ	ERα Exon 7Δ	$ER\beta_1$	$ER\beta_2$
MCF-7	$2.4x10^7$	$9x10^5$	$1.6x10^5$	$6x10^5$	$1.4x10^5$	$1.8x10^6$	0	$1.2x10^6$	$2.5x10^5$	$3.5x10^5$
T47D	$6x10^7$	$2.5x10^6$	$2.6x10^5$	$1.2x10^6$	$1.2x10^6$	$6.1x10^6$	0	$2.5x10^6$	$4.0x10^5$	$1.6x10^4$
ZR-75	$7 x10^6$	$1.2x10^6$	$3.5x10^5$	$4.1x10^5$	$2.0x10^5$	$8.0x10^5$	0	$5x10^5$	$4.0x10^5$	$4.0x10^4$

TABLE 2

MESSENGER RNA RATIOS OF ERα WILD TYPE TO OTHER ER ISOFORMS IN MCF-7, T47D AND ZR-75 CELL LINES

Cell Type	ERα Exon 2Δ	ERα Exon 2-3Δ	ERα Exon 3Δ	ERα Exon 4Δ	ERα Exon 5Δ	ERα Exon 6Δ	ERα Exon 7Δ	$ER\beta_1$	$ER\beta_2$
MCF-7	0.04	0.006	0.025	0.006	0.08	0	0.05	0.01	0.014
T47D	0.04	0.004	0.02	0.02	0.1	0	0.04	0.006	0.0002
ZR-75	0.17	0.05	0.05	0.02	0.1	0	0.07	0.06	0.006

Figure 1

Scatchard Plots Of Estrogen Binding To MCF-7, T47D and ZR-75 Extracts

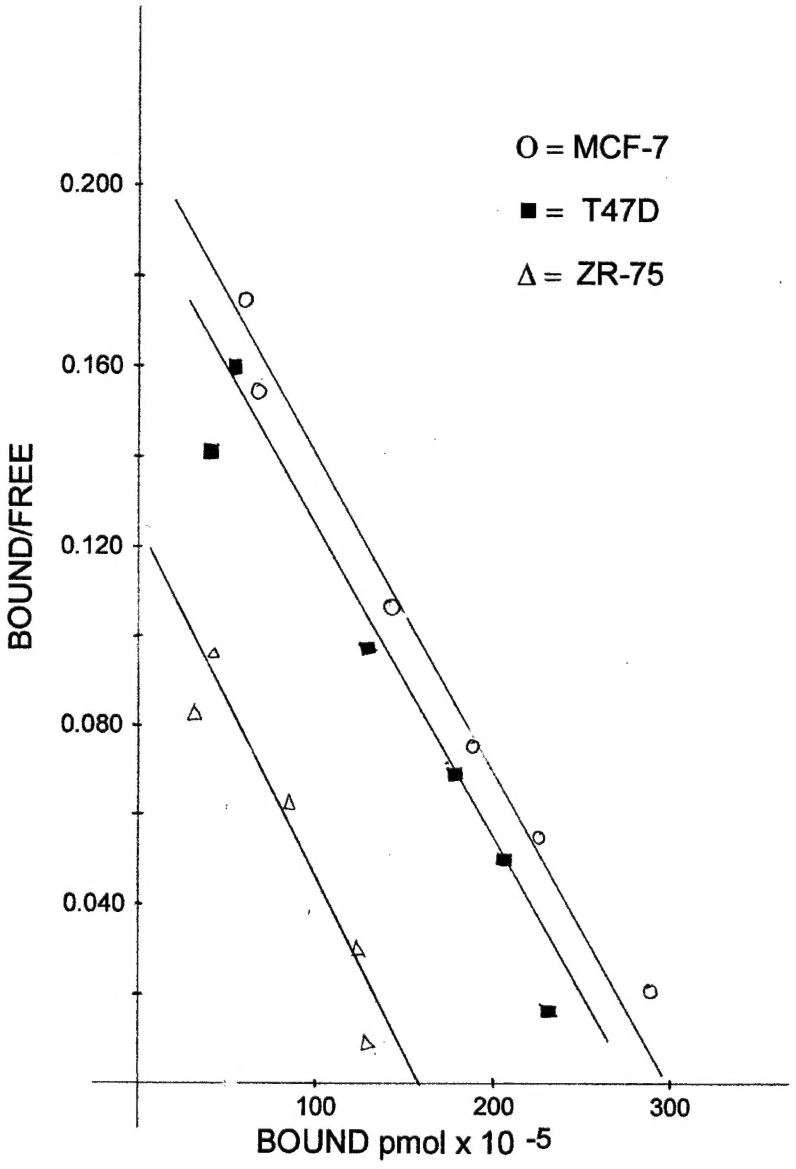
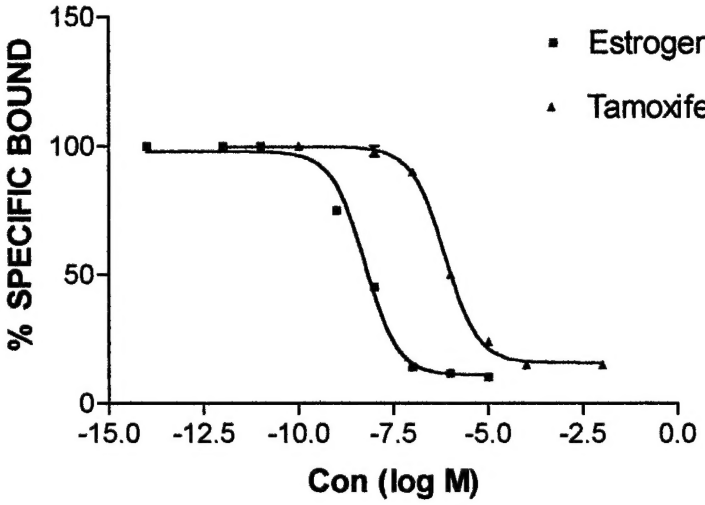


Figure 2

Relative Affinities of Estrogen and Tamoxifen to MCF-7, T47D and ZR-75 Breast Cancer Cell Lines

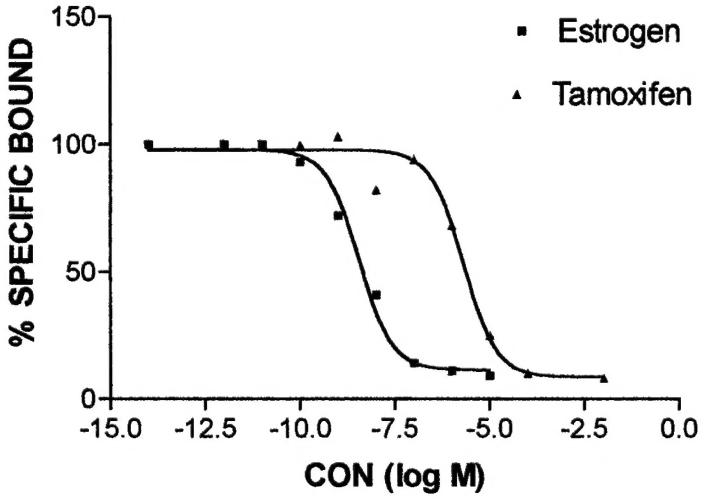
MCF-7



$IC_{50}=5.6x10^{-9}$

$IC_{50}=7.2x10^{-7}$

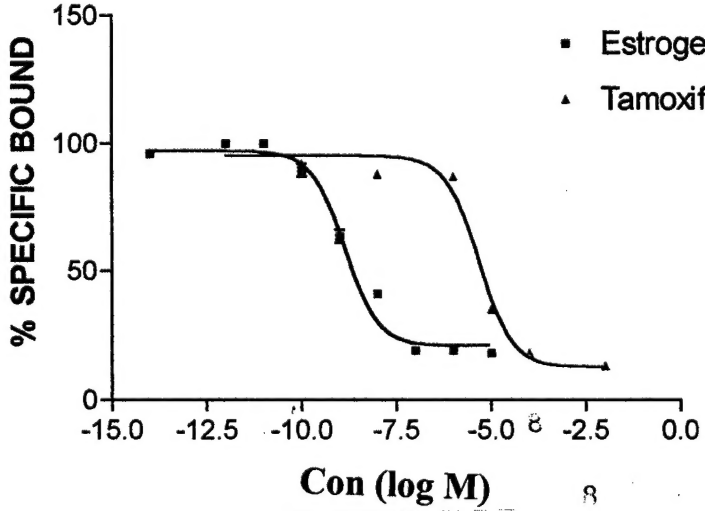
T47D



$IC_{50}=3.6x10^{-9}$

$IC_{50}=2.1x10^{-6}$

ZR-75



$IC_{50}=1.4x10^{-9}$

$IC_{50}=4.8x10^{-6}$

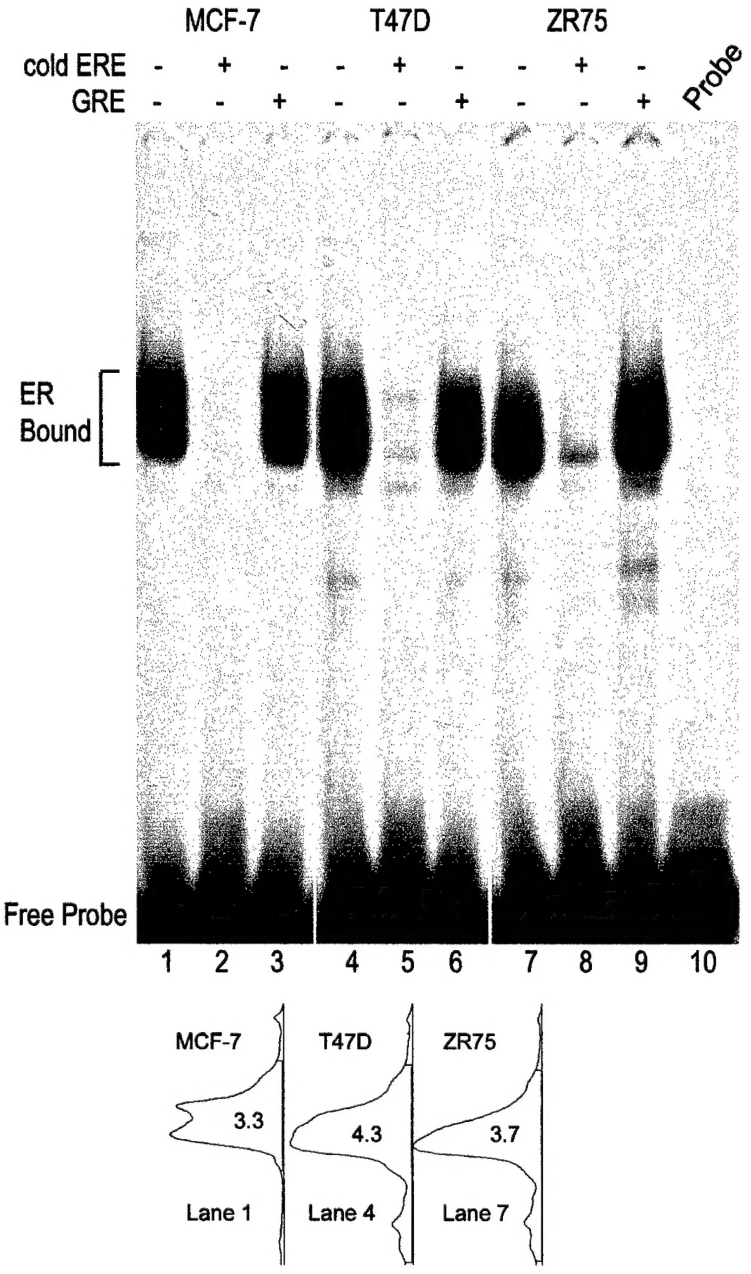
TABLE 3

RELATIVE BINDING AFFINITIES OF ESTROGEN AND TAMOXIFEN FOR MCF-7, T47D AND ZR-75

Cell Type	Estrogen	Tamoxifen	Relative Binding Affinity of Estrogen to Tamoxifen
MCF-7	5.6×10^{-9} M	7.2×10^{-7} M	8.0×10^{-3}
T47D	3.6×10^{-9} M	2.1×10^{-6} M	1.7×10^{-3}
ZR-75	1.4×10^{-9} M	4.8×10^{-6} M	2.9×10^{-4}

Figure 3

Mobility Shift Assay to Determine DNA Binding of Estrogen Receptors in Cell Extracts



Density scans of Lanes 1, 4, and 7 with the relative areas indicated

Responsivness to Estrogen

In order to determine the biological response of estrogen receptor stimulation by estrogen and or tamoxifen, we looked for induction of progesterone receptor by estrogen treatment. Cultured cells which had been grown in estrogen-free conditions were exposed to estrogen for 36 hours and then were harvested and run on SDS-PAGE gels. The gels were blotted and stained with antibodes to progesterone receptor. These experiments are still in the prelimnary stages and a significant increase in progesterone receptor has been seen only with the ZR75 cells.

KEY RESEARCH ACCOMPLISHMENTS

- The three cell lines studied all have distinct profiles of estrogen receptor mRNAs
- T47D had the highest total ER mRNA
- ZR75 had the highest relative amounts of ERα splice variants
- All three cell lines had different IC_{50} values (affinity) for tamoxifen
- ZR75 had the highest relative resistance to tamoxifen

REPORTABLE OUTCOMES - None

CONCLUSIONS

We have found distinct populations of ERs in three cell lines and distinct responses to the anti-estrogen, tamoxifen. We need to examine more cell lines to determine which ER isoforms out of the total population are significant in determining cell response to estrogens and anti-estrogens. We need to do this both with estrogen and anti-estrogen binding studies and with studies of induction of progesterone receptor. If we are able to determine that a particular set of ER isoforms is important in response or resistance to tamoxifen or other anti-estrogens, this may help determine beforehand which cancer patients will benefit from anti-estrogen therapy.

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